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Original article

Pharmacokinetics and pharmacodynamics of a single dose of sustained-release azithromycin formulation in pigeons

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Abstract

To date, only a few studies on the azithromycin (AZM) pharmacokinetics in ornamental birds have been published. In the current study AZM concentrations in domestic pigeon (*Columba livia domestica*) plasma samples were analyzed using a validated ultra-high performance liquid chromatography tandem mass spectrometry method. The aim of the current study was to carry out an analysis of the pharmacokinetics and pharmacodynamics after administration of a single oral dose of a sustained-release AZM formulation and to conduct a simulation of treatment based on selected minimal inhibitory values. The study was performed with 12 healthy adult pigeons, both sexes. The pigeons tolerated AZM very well and no adverse effects were observed in any animal during the study. Based on the observed characteristics of the pharmacokinetics/ /pharmacodynamics profiles of AZM in pigeons, it should be noted that 35 mg/kg *per os* as a single starting dose and 25 mg/kg every 24 h are recommended for treatment of both susceptible and less susceptible pathogens.

Key words: azithromycin, pigeon, pharmacokinetics, pharmacodynamics, dose

Introduction

Azithromycin (AZM) is a macrolide antibiotic used for oral administration in human and veterinary medicine. AZM is classified as a long half-life drug (elimination half-life >24h) with very good distribution into tissue compartments. In humans, its concentrations in lung tissue or tonsils could be >100× higher than in serum and 18-38× higher in the murine model, depending on the formulation (FDA 2009, Rivulgo et al. 2013). High levels of AZM were demonstrated in peripheral blood mononuclear cells (PBMCs) and polymorphonuclear cells (PMNs). The intracellular concentration in that case could be >30× higher than

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in the extracellular compartment (Gladue et al. 1989, Gladue and Snider 1990). Moreover, the unchanged drug takes part in enterohepatic recirculation. In humans, 50% of unchanged AZM is eliminated with feces (Luke and Foulds 1997).

Azithromycin is transported with PBMCs and PMNs across the body. This uptake and transport is not saturable and plays an important role in the distribution, elimination, and clinical efficacy of the drug (Sampson et al. 2014). Taking into account all AZM features, new strategies of dosage and treatment in humans have been proposed. Moreover, current human studies have shown that a single-dose regimen could be as effective as a multiple dose treatment for several days (Gordon and Blumer 2004, Law and Amsden 2004, Blumer 2005, Girard et al. 2005) . In veterinary medicine, this is a special topic due to the possible environmental contamination by feces containing unchanged drug and growing antibiotic resistance.

To date, only a few studies on the AZM pharmacokinetics in ornamental birds have been published (Limoges et al. 1998, Carpenter et al. 2005). Carpenter and coworkers have published data from investigations of *Ara ararauna* after intravenous (IV) and oral administration (PO) of AZM (Carpenter et al. 2005). Limoges and coworkers have described the PO pharmacokinetics in *Amazona farinosa* (Limoges et al. 1998). IV and PO pharmacokinetics in the broiler chicken has also been described (Abo-El-Sooud et al. 2012). Guzman and coworkers have shown the efficacy of the drug in a study of *Nymphicus hollandicus* exposed to 21-day treatment (Guzman et al. 2010). The efficacy and *in vitro* synergistic effect of baicalin with AZM in *Staphylococcus saprophyticus* isolated from *Francolinus* spp. have been described by Wang and coworkers (Wang et al. 2018). In the case of breeding birds, data on the prevalence of antimicrobial resistance to AZM in *Campylobacter* spp. from broiler chickens have been published by Li and coworkers (Li et al. 2017). Administration of a drug *per os* is associated with stress in birds, as they have to be repeatedly captured. Therefore, the choice of an antimicrobial agent characterized by sustained release is highly advisable. However, no research related to pharmacokinetics, pharmacodynamics, and treatment regiments in pigeons (*Columba livia domestica*) has been published so far.

The aim of the current study was to carry out an analysis of the pharmacokinetics and pharmacodynamics after administration of a single oral dose of a sustained-release AZM formulation and to conduct simulation of treatment based on selected minimal inhibitory values.

Materials and Methods

Animals

The study was performed with 12 healthy adult pigeons (*Columba livia domestica*), both sexes (1:1), with body weight of 380-510 g. The pigeons were obtained from breeding (Zawadówka, Poland). The study protocol was approved by the ethics committee of the University of Life Sciences, Lublin (78/2015). All animals underwent a 2-week acclimatization period. The pigeons were deprived of food 2 h prior to drug administration, and 1 h after the treatment, water was available *ad libitum*. The pigeons were administered 10 mg/kg AZM (Zetamax® 2g, sustained-release granules for oral suspension, Pfizer®) by oral application via a gavage tube (Carpenter et al. 2005). Adverse effects were monitored within the study based on clinical examination by veterinary doctor. After the drug administration, blood was sampled from the left or right ulnar vein with 25G needles (KDM, KD-FINE) into heparinized tubes. 0.5 mL of blood was taken at time 0 before the administration and at intervals of 0.5, 1, 3, 6, 12, 24, 48, and 72 h after the treatment. The blood samples were placed in a prelabeled tube and centrifuged for 8 min at 2000 × *g*. Blood plasma was collected and frozen at -20°C until analysis. The blood plasma AZM concentrations were subsequently analyzed by ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS).

Analytical method

In the current study AZM concentrations in pigeon plasma samples were analyzed with a validated UHPLC-MS/MS method. All solvents used in the method for analysis of AZM in pigeon plasma were analytical grade. Analytical standards of AZM and josamycin used as an internal standard (IS) were obtained from Sigma Aldrich Chemical (USA). Frozen samples of pigeon plasma were thawed, mixed, and centrifuged. A 200 µL aliquot of the plasma was mixed with the IS. 800 µL of 0.1% formic acid in acetonitrile were then added, vortexed, and centrifuged at 13000 × g. The supernatant was then filtered through 0.22 hydrophilic polyvinylidene fluoride syringe filters into autosampler vials. The UHPLC-MS/MS analysis of the AZM concentrations in the plasma were determined using a Shimadzu Nexera X2 (Shimadzu, Japan) system connected to a QTRAP® 4500 triple quadrupole mass spectrometer (AB Sciex, USA). The multiple reaction monitoring mode in the positive ion electrospray ionization mode was used for detection. To monitor AZM, two transitions 749→591.0 *m/z* and 749→58.4 *m/z* were applied. The transition of 828→173.9 was used

for IS. The MS/MS system was set at the following conditions: declustering potential (DP) 89 V, collision energy (CE) ion 1, 40 V and ion 2, 53 V, and cell exit potential (CXP) 13V for AZM and DP 80V, CE 15V, CXP 46V for IS. Analyst 1.6.3 software controlled the UPLC system. The chromatographic analysis was performed on a ZORBAX SB-C18 50×2.1 mm × 1.8 µm (Agilent, USA) analytical column with a C18 guard column 4×2 mm (Phenomenex, USA). The column was maintained at 35°C. The mobile phase consisted of 0.1% formic acid (A) and acetonitrile (B) in a gradient mode. The following program was set: 0-1.3 min: 100% A; 1.31-3.0 min: 50% A; 3.01-5.0 min: 100% A. The mobile phase flow rate was 0.6 mL/min. The injection volume was established as 5 µL.

The method was validated according to current guidelines (EMA 2011; FDA 2018). The study was performed in compliance with the principles of good laboratory practice (GLP). The validation study was realized in terms of linearity, specificity, precision (repeatability, within-laboratory reproducibility), and recovery. The precision was evaluated at three concentration levels of 5, 20, and 50 ng/mL (n=6), from run to run during 1 day for repeatability and 3 days for within-laboratory reproducibility. The precision was calculated at each fortification level by evaluating the relative standard deviation of the obtained results. The recovery was evaluated in the same test as precision by comparison of the mean concentrations with the concentrations of AZM in fortified samples. The matrix effect was also evaluated by comparing the peak area of the analyte in the solvent (set 1) and the peak area after fortifying the analyte of extracted blank plasma (set 2) at a 5 ng/mL concentration level (n=5) after injection on the UHPLC-MS/MS instrument. The matrix effect was specified as the response of samples of set 2 to samples of set 1, expressed in percentage. The value of 100% indicates no matrix effect, a value above 100% ion shows enhancement, and a value below 100% reflects ion suppression.

Pharmacokinetic and pharmacodynamic parameters

Pharmacokinetics analysis was done using a Phoenix 64 WinNonlin version 8.0 (Certara LP). Due to the low plasma volume available for sampling, sparse sampling was used in the study. The sparse sampling model with noncompartmental analysis (NCA) was used for all pharmacokinetics parameters without the absorption rate constant (tvk_a) and between compartmental rate constant analysis. The sparse NCA approach was used for: k_{el} – elimination rate constant; $t_{1/2kel}$ – elimination half-life; Span – number of $t_{1/2kel}$ between ranges used for calculation of k_{el}; t_{max} – time of the maximum concentration; C_{max} – maximal concentration; t_{last} – time of the last concentration observed; C_{last} – last concentration observed; $AUC_{0\text{-}tlast}$ – area under the curve between time 0 and the last concentration observed; $AUC_{0\text{-}inf}$ – area under the curve between time 0 and infinity; $AUC_{\%}$ – extrapolated area under the curve after t_{last}; V_F – volume of distribution; Cl_F – total body clearance; $AUMC_{0\text{-}inf}$ – area under the first moment curve between time 0 and infinity; $MRT_{0\text{-}inf}$ – mean residence time based on $AUC_{0\text{-}inf}$ and $AUMC_{0\text{-}inf}$. In the case of sparse sampling, only linear trapezoidal methods for AUC calculations were available. k_{el} was calculated with uniform weighting. Since the sparse NCA method did not support the tvk_a analysis, a population approach was used for estimation of tvk_a with an approach presented before (Grabowski et al. 2018). Based on sparse data, selected parameters related to the between compartment rate of distribution were calculated: k_{12} – rate constant between the central and tissue compartment; k_{21} – rate constant between the tissue and central compartment; k_{10} – elimination rate constant from the central compartment; k_{20} – elimination rate constant from the tissue compartment. The analysis was done using mean values of sparse data, stripping analysis, a two-compartment model, and using ThothPro 4.1 software (ThothPro LLC). The model was selected based on Akaike information criterion. Based on the PK analysis and PK/PD data, a scheme of multiple dose and dosing intervals were proposed using ThothPro 4.1 software (ThothPro, LLC). A treatment scenario with appropriate τ – dosing intervals was proposed within 6 days of treatment based on linear extrapolation of observed data and a single dose pharmacokinetic profile.

The analysis was performed based on the minimal inhibitory concentration for 50% population (MIC_{50}) for selected strains of bacteria detected in pigeons and other birds: *Borrelia burgdorferi* (Hunfeld et al. 2000; Raele et al. 2018)*; Legionella pneumophila* (Fabbi et al. 1998, Yamaguchi 2001) *Moraxella catarrhalis* (Mattsson et al. 1999, Rittenhouse et al. 2006); *Erysipelothrix rhusiopathiae* (Soriano et al. 1998, Cousquer 2005, Bobrek and Gawel 2015)*; Chlamydia psitttaci* (Donati et al. 1999), *Camphylobacter* spp. (Noormohamed and Fakhr 2014), *Chlamydia trachomatis* (Donati et al. 1999), *Streptococcus gallolyticus* (Haas et al. 2009), and *Chlamydia pneumoniae* (Donati et al. 1999). PK/PD were calculated by Phoenix 64 WinNonlin version 8.0 (Certara L.P.). Time above MIC_{50} and area under the curve after a single dose ratio to MIC_{50} are key predictors of the clinical efficacy of AZM (Van Bambeke and Tulkens 2001). Consequently, the calculated parameters include $T>MIC_{50}$ – total time

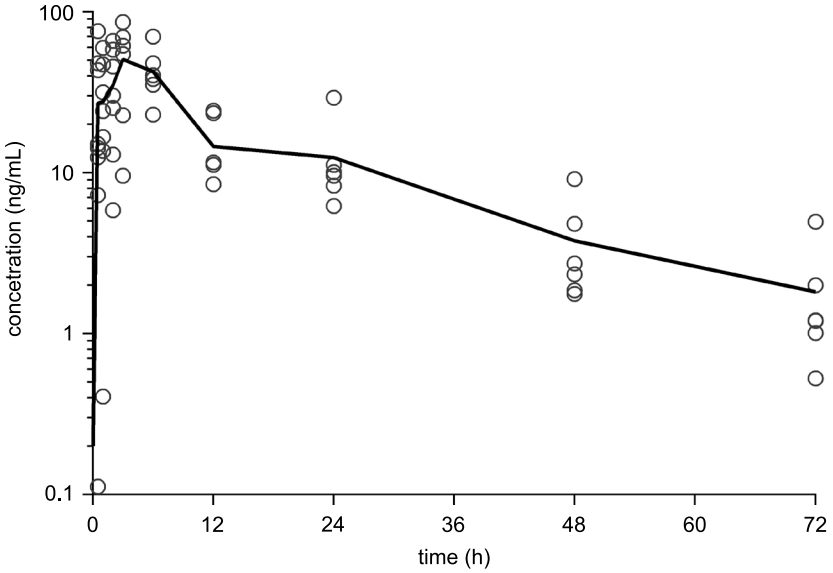

Fig. 1. Pharmacokinetic profile in blood plasma after a single 10 mg/kg body weight dose of AZM in healthy pigeons (*Columba livia domestica*). Gray rings – observed concentrations for each time point; black line – arithmetic mean value.

above MIC_{50}, [h] as nominal time, [%] as a percent of the dose interval equal to 24h; $AUC_{0\text{-}24}/MIC_{50}$ – area under the curve between time 0 and 24 h ratio to the minimal inhibitory concentration.

Statistical analysis

Standard error (SE) of the mean C_{max} was calculated as the sample standard deviation of the y-values at t_{max} divided by the square root of the number of observations at t_{max}. SE for AUC was calculated as described by Nedelman and Jia with Holder modification (Nedelman and Jia 1998, Holder 2001). Based on observed data, the C_{max} and C_{last} standard deviation and between animal variability were calculated as a percent of relative standard deviation (RSD%).

Results

The pigeons tolerated AZM very well and no adverse effects were observed in any animal during the study. The blood plasma concentrations of AZM following a dose of 10 mg/kg BW after PO administration are shown in Fig. 1. With the sparse sampling methodology, SE was reported only in the case of C_{max} and AUC. The results of noncompartmental and population analysis are presented in Table 1. The between animal variability of C_{max} was >52 RSD% and C_{last} >81 RSD%. AZM was detected in plasma up to 72 h with substantially lower $AUC_{\%}$ than 20%. The data represent a two-compartment open model with first-order absorption. Akaike information criterion for one and two-compartment models were 34.9 and 30.01 respectively. The tvk_a value was equal to 0.0988 h^{-1} with 15.09 CV%. k_{12} (0.007 h^{-1}) was 8.7-fold lower than k_{21} (0.061 h^{-1}) and k_{10} (0.054 h^{-1}) was 8.4-fold lower than k_{20} (0.451 h^{-1}).

The PK/PD analysis was performed for a few indicative MIC_{50} values. The PK/PD parameters related to the MIC_{50} values of AZM following a 10 mg/kg BW PO administration are shown in Table 2. Based on these data and the linear PK profile between 10-50 mg/kg, $AUC_{0\text{-}24}/MIC_{50}$ was calculated for any MIC_{50} values and dose using the equation below:

$$AUC_{0\text{-}24}/MIC_{50} = 56.867 \times Dose \times [1/MIC_{50}] \quad (R^2 = 1), \text{Eq.1}$$

It was calculated that a dose of 15 mg/kg is needed to achieve T_{inf}>MIC equal to 24h for susceptible pathogens (MIC_{50}= 0.015 µg/mL). Such a level of this parameter is not available for MIC_{50} between 0.03-0.125 µg/mL for doses 10-20 mg/kg and for MIC_{50} 0.125 µg/mL in the case of a dose of 50 mg/kg. Prediction of multiple dose profiles needs a 25 mg/kg dose every 24 h to exceed the 0.125 µg/mL level. To exceed this level immediately after the first dose, a 35 mg/kg starting dose was required (Fig. 2).

The developed UHPLC-MS/MS method was linear (r^2>0.99) in the concentration range of 0.1–100 ng/mL. In the specificity test, no interferences were recorded at the retention time of the target analyte. The validation results showed good precision with RSD less than 8% for repeatability and less than 14% for within-laboratory reproducibility. Depending on the validation level, the recovery was calculated as 98.4, 99.1, and 100.8%. The procedure was satisfactorily sensitive with a lower limit of quantitation of 0.1 ng/mL. No significant matrix effect was observed for AZM in the presented method (104%).

Table 1. Pharmacokinetic parameters of AZM after a single 10 mg/kg body weight dose in healthy pigeons (*Columba livia domestica*).

Parameters	Unit	M; (SE); |SD|
k_{el}	h^{-1}	0.0369
Span	na	3.200
$t_{1/2kel}$	h	18.744
t_{max}	h	3
C_{max}	ng/mL	50.83; (11.90);|26.62|
t_{last}	h	72
C_{last}	ng/mL	1.819;|1.474|
$AUC_{0\text{-}tlast}$	h×ng/mL	830.68; (124.21)
$AUC_{0\text{-}inf}$	h×ng/mL	878.16
$AUC_{\%}$	%	5.407
V_F	L/kg	307.94
Cl_F	L/h/kg	11.387
$AUMC_{0\text{-}inf}$	h^2×ng/mL	19310.62
$MRT_{0\text{-}inf}$	h	21.946

M – arithmetic mean; SE – standard error; SD – standard deviation; kel – elimination rate constant; $t_{1/2kel}$ – elimination half-life; Span – number of $t_{1/2kel}$ between ranges used for k_{el} calculation; tvk_a – typical value of absorption rate constant; t_{max} – time of the maximum concentration; C_{max} – maximal concentration; t_{last} – time of the last concentration observed; C_{last} – last concentration observed; $AUC_{0\text{-}tlast}$ – area under the curve between time 0 and the last concentration observed; $AUC_{0\text{-}inf}$ – area under the curve between time 0 and infinity; $AUC_{\%}$ – extrapolated area under the curve after t_{last}; V_F – volume of distribution; Cl_F – total body clearance; $AUMC_{0\text{-}inf}$ – area under the first moment curve between time 0 and infinity; $MRT_{0\text{-}inf}$ – mean residence time based on $AUC_{0\text{-}inf}$ and $AUMC_{0\text{-}inf}$.

Table 2. Pharmacokinetic/pharmacodynamic parameters of AZM in healthy pigeons (*Columba livia domestica*) based on MIC_{50} values for selected pathogenic microbial strains.

Parameters/Dose	1	2	3	4	5
MIC_{50} [mg/mL]	0.015	0.016	0.03	0.06	0.125
Dose 10 mg/kg					
T>MIC [h]	11.64	11.41	7.38	0.00	0.00
T>MIC [%]	48.51	47.54	30.73	0.00	0.00
$AUC_{0\text{-}24}/MIC_{50}$ [h]	37911.35	3554.19	18955.68	9.48	4.55
Linear extrapolation for 20 mg/kg single dose					
T>MIC [h]	37.57	36.18	11.64	7.38	0.00
T>MIC [%]	156.54	150.74	48.51	30.73	0.00
$AUC_{0\text{-}24}/MIC_{50}$ [h]	75822.70	7108.38	37911.35	18.96	9.10
Linear extrapolation for 50 mg/kg single dose					
T>MIC [h]	57.49	55.04	41.75	25.04	9.31
T>MIC [%]	239.56	229.34	173.95	104.32	38.78
$AUC_{0\text{-}24}/MIC_{50}$ [h]	189556.75	17770.95	94778.38	47.39	22.75

T>MIC – total time above MIC_{50}, [h] as nominal time, [%] as a percent of the dose interval equal to 24h; $AUC_{0\text{-}24}/MIC_{50}$ – area under the curve between time 0 and 24 h ratio to the minimal inhibitory concentration; 1-5 – range of studied MIC_{50} based on selected examples of pathogens; 1 – *Borrelia burgdorferi* (Hunfeld et al. 2000); 2 – *Legionella pneumophila* (Yamaguchi 2001), *Moraxella catarrhalis* (Rittenhouse et al. 2006); 3 – *Erysipelothrix rhusiopathiae* (Soriano et al. 1998); 4 – *Chlamydia psittaci* (Donati et al. 1999), *Campylobacter spp.* (Noormohamed and Fakhr 2014), *Chlamydia trachomatis* (Donati et al. 1999), *Streptococcus bovis* (Haas et al. 2009); 5 – *Chlamydia pneumoniae* (Donati et al. 1999).

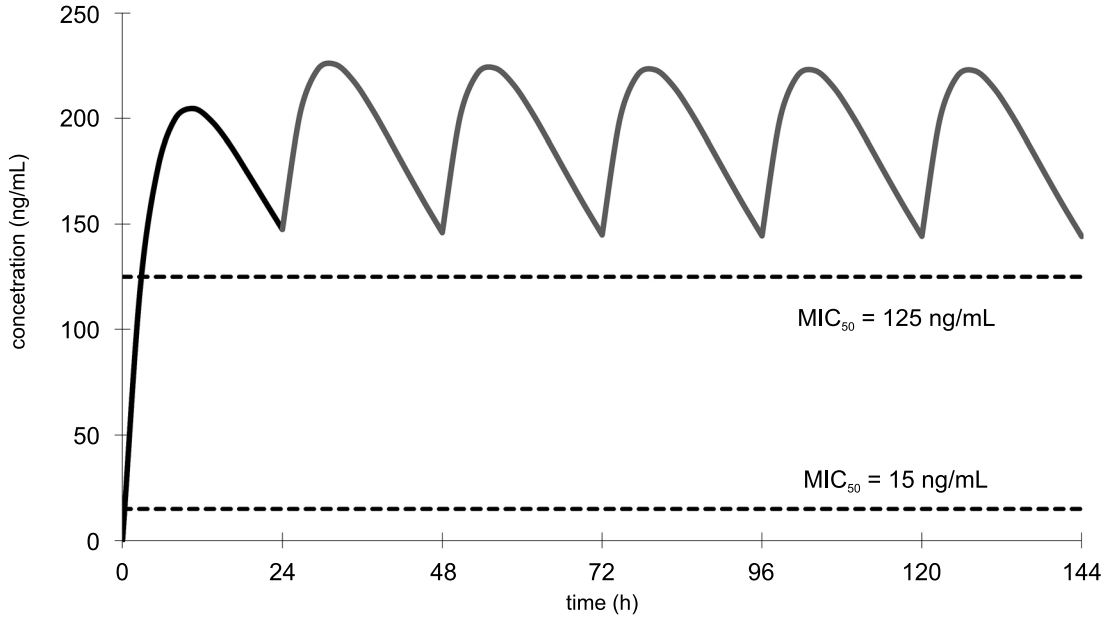

Fig. 2. Simulation of PK profiles after multiple oral AZM treatment in pigeons. Black line – single 35 mg/kg starting dose; shadow line – regular dose, 25 mg/kg every 24 h.

Discussion

As AZM is a widely used antibiotic in human and veterinary medicine, many researchers investigate it with highly sensitive chromatographic methods. Some HPLC methods for AZM detection reported in the literature were based on fluorometric detection following derivatization of AZM (Wilms et al. 2005). However, as the derivatization process is labor-intensive, LC-MS/MS has become the method of choice for quantitation of AZM in various biological matrices (Sharma and Mullangi 2013). In the method presented in this study, a fast, simple, and environmentally friendly sample preparation procedure was used, allowing parallel processing of a large number of samples.

Only a few papers have been devoted to the PK of AZM in ornamental birds. The pharmacokinetic profile of AZM in pigeons represents two-compartment distribution, which shows the same scheme as in other species and humans. Compared to macaws, the AZM clearance in pigeons is more than 15 th fold faster (Carpenter et al. 2005). However, it can certainly be claimed that the pharmacokinetic profile of AZM in pigeons is not as long as in the case of other birds. The $t_{1/2kel}$ and MRT of AZM in pigeons are shorter than 24 h, which is not a typical value for birds and other animals (Limoges et al. 1998, Carceles et al. 2005, Carpenter et al. 2005, Abo-El-Sooud et al. 2012, Leclere et al. 2012, Zur et al. 2014). The shorter half-life could be a consequence of faster clearance, and a high basal metabolic rate (BMR) in pigeons close to the BMR of rats (Montgomery et al. 2011).

Similar to other animals and humans, a slight *plateau* between 12 and 24 h after administration could be a consequence of enterohepatic recirculation. Interestingly, despite the long $t_{1/2kel}$, the rate of transfer from the tissue to the central compartment is much faster than from blood to tissue (k_{21}/k_{12}). This comparison indirectly confirms the important role of AZM recirculation with bile in maintaining the relatively long $t_{1/2kel}$ in pigeons. The same relationship characterizes the (k_{20}/k_{10}) ratio. The whole PK profile indicates high between animal variability related to the complex nature of AZM disposition in the body. However, the between animal variability of C_{max} in the pigeons was 20% lower than after oral administration of the same dose in macaws (Carpenter et al. 2005). The calculated volume of distribution was more or less ten times higher in pigeons than macaws. This is related to the different character of both PK curves. In the case of macaws, the concentration-time profile (C-T) is flatter and results in a higher MRT value.

In the case of AZM, the key PK/PD parameters representing $T>MIC_{50}$ and AUC_{0-24}/MIC_{50} could be important for clinical efficacy (Nightingale 1997). The expected value for AUC_{0-24}/MIC_{50} should exceed 125 (Van Bambeke and Tulkens 2001). Based on Equation 1, it can be assumed that MIC_{50} values that give $AUC_{0-24}/MIC_{50}>125$ in pigeons are MIC_{50}=0.022 µg/mL for the dose of 50 mg/kg or MIC_{50}=0.045 µg/mL for the dose of 50 mg/kg. $T>MIC_{50}$ [%] close to 50% of τ is sufficient to achieve maximal bactericidal activity (Craig 1996). The current study clearly indicates that in pigeons this is possible only in the case of doses of 20 mg/kg and higher. On the other hand, due to the significant post-antibiotic effect of AZM

in vivo, the AUC_{0-24}/MIC_{50} value is an important parameter of PK/PD from the clinical point of view (Van Bambeke and Tulkens 2001).

In a study conducted by Carpenter and coworkers, doses of 10 mg/kg PO every 48 h for susceptible bacteria in macaws were recommended (Carpenter et al. 2005). Given the 5-fold higher C_{max} and 3-fold longer MRT in macaws than pigeons after a 10 mg/kg PO dose, this scheme sounds reasonable. In human medicine, new trends in AZM treatment suggest a single dose instead of multiple treatment (Gordon and Blumer 2004, Law and Amsden 2004, Blumer 2005, Girard et al. 2005). Due to the long $t_{1/2kel}$ of AZM in humans, such a strategy is possible for implementation in clinical practice (FDA 2009). Unfortunately, the relatively short $t_{1/2kel}$ and fast clearance do not allow implementation of such an approach in the treatment of pigeons. The single dose approach in pigeons would require very high doses. Bearing in mind that a significant part of macrolides is excreted in an unchanged form with feces, high doses would lead to environmental contamination and enhancement of microbial resistance. Consequently, based on the observed characteristics of the C-T profiles of AZM in pigeons, it should be noted that 35 mg/kg PO as a single starting dose and 25 mg/kg every 24 h are recommended for treatment of both susceptible and less susceptible pathogens.

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